

(19)

Europäisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 1 002 542 A1

(12)

EUROPEAN PATENT APPLICATION

published in accordance with Art. 158(3) EPC

(43) Date of publication:
24.05.2000 Bulletin 2000/21

(51) Int. Cl.⁷: **A61K 38/22**

(21) Application number: **99913324.2**

(86) International application number:
PCT/ES99/00101

(22) Date of filing: **16.04.1999**

(87) International publication number:
WO 99/53944 (28.10.1999 Gazette 1999/43)

(84) Designated Contracting States:
**AT BE CH CY DE DK FI FR GB GR IE IT LI LU MC
NL PT SE**

(30) Priority: **17.04.1998 ES 9800814**

(71) Applicant:
**UNIVERSIDAD COMPLUTENSE DE MADRID
E-28040 Madrid (ES)**

(72) Inventors:
• **PEREZ GOMARIZ, Rosa,
Facultad de Ciencias
E-28040 Madrid (ES)**

- **LECETA MARTINEZ, Javier,
Facultad de Ciencias
E-28040 Madrid (ES)**
- **DELGADO MORA, Mario,
Facultad de Ciencias
E-28040 Madrid (ES)**
- **MARTINEZ MORA, Carmen,
Facultad de Ciencias
E-28040 Madrid (ES)**

(74) Representative:
**Carpintero Lopez, Francisco
HERRERO & ASOCIADOS, S.L.
Alcalá, 21
28014 Madrid (ES)**

(54) METHOD FOR TREATING THE ENDOTOXIC SHOCK IN MAMMALS

(57) Disclosed is the use of vasoactive intestinal peptide (VIP) and the peptide activator of the hypophyseal adenylyl cyclase (PACAP) in the treatment of the endotoxic shock in mammals. These substances inhibit the production of the tumoral necrosis factor (TNF) and of interleukine 6 (IL-6).

EP 1 002 542 A1

Description

STATE OF THE ART

[0001] Endotoxic shock is still the main cause of death in hospital. Strategies for combating the effects of endotoxic shock are centred on counteracting the bacterial agents responsible for these effects, restoring the hemodynamic parameters, preventing cellular activation and modifying the action of the defence mechanisms (Boyd O; *Current Opinion in Anaesthesiology* 1996, 9:98)

[0002] It is currently accepted that the inflammatory response to bacterial products directly contributes to endotoxic shock (Parillo JE; *New England Journal of Medicine* 1993, 328: 1471). Toxic bacterial products and those released during tissue damage activate the defence mechanisms, with implication of cells such as neutrophils, monocytes, macrophages and endothelial cells, and of mediators such as cytokines, platelet activation factor, metabolites of arachidonic acid and nitric oxide, leading to hemodynamic changes and organ lesions for the host (Moldawer LL; *Critical Care Medicine* 1994, 22: 3). Many cytokines have been proposed as markers of the seriousness of the development of septic shock. The levels of circulating TNF-, IL-1, IL-6 and IL-8 have been correlated with the probability of overcoming a septic episode. TNF-, and IL-1 administered to humans or experimental animals reproduce many of the hemodynamic signs of septic shock (Tracey KJ et al.; 1986, *Science* 234:470). Their inhibition by injection of antagonist receptors and blocking monoclonal antibodies have been studied with a wide range of results (Fisher CJ et al.; 1994, *Critical Care Medicine*, 22: 12). Of the immunological markers the levels of circulating IL-6 are the best indicators of the seriousness of the sepsis and the possibilities of overcoming the episode (Liaw YS et al.; 1997, *Journal of the Formosan Medical Association*, 96:685). Despite the advances in knowledge of the mechanisms and of the technical and pharmacological progress there are still few results in terms of an improvement in the data for mortality rate. This rate corresponds to 200,000 deaths per year in the United States and Europe (Vicent J-L and Chamliou R; *Current Opinion in Anaesthesiology* 1996, 9:146).

[0003] The Vasoactive Intestinal Peptide (VIP) is a basic peptide of 28 amino acid units whose sequence is (Mutt V and Said SI; *European Biochemistry* 1974, 42:581):

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH₂

It was initially isolated from the small intestine of pig and later identified in the brain and terminals of the peripheral nervous system. It was established to be a neu-

ropeptide with neuromodulating properties (Fahrenkrug J; *Pharmacology and Toxicology* 1993, 72:354). Its name is derived from its peripheral vasodilatory properties. VIP has also been identified in rat mast cells and in granulomas (Cutz E. et al; *Nature* 1978, 275:661). Immunological studies carried out on histologic sections of thymus, spleen and lymphatic ganglia from rats have identified immunoactive VIP in the lymphocytes of these organs (Leceta et al. *Advances in Neuroimmunology* 1996, 6:29). VIP exercises its biological effects through membrane receptors belonging to the super family of seven hydrophobic domains coupled to G proteins, which transduce information to the end effector molecules (Laburthe M y Couvineau A; *Annals of the New York Academy of Sciences* 1988, 527:296). Receptors for VIP have been characterised in several tissues such as liver and adipose tissue among others. These correspond to two types, the so-called VIP1-R (Ishihara T et al.; *Neuron* 1992, 8:811) and VIP2-R (Lutz E. et al. *FEBS Letters* 1993, 334:3). In the immune system specific receptors have been characterised for VIP in a variety of immune cells which include human peripheral lymphocytes, human monocytes, rat and mouse lymphocytes, rat alveolar macrophages and peritoneal macrophages of rat and mouse (Delgado M et al.; *Regulatory Peptides* 1996, 62:161). VIP modulates a great variety of immune functions such as phagocyte function, at every stage of the process, the proliferative response, production of immunoglobulin, NK activity and cytokine production (Ganea et al.; *Advances in Neuroimmunology* 1996, 6:61).

[0004] The peptide activator of hypofissary adenylate cyclase (PACAP) is a member of the family of peptides of secretin/VIP/glucagon, of which two molecular forms are known, namely PACAP-38 and PACAP-27, whose sequences are as indicated (Ogi K et al.; *Biochemical and Biophysical Research communication* 1993, 196:1511):

PACAP-38

His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Thy-Leu-Ala-Ala-Val-Leu-Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH₂

PACAP-27

His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Thy-Leu-Ala-Ala-Val-Leu-NH₂

[0005] Both peptides are widely distributed in the central and peripheral nervous system. There are also PACAP producing cells in lung, pancreatic B cells and intestine (Arimura A; *Regulatory Peptides* 1992, 37:287). In the immune system a large abundance of PACAP positive cells have been described in central and peripheral lymphoid organs (Gaytan F et al.; *Cell and Tissue Research* 1994, 276:233). For PACAP three types of receptor have been described (Shivers BD et

al.; Endocrinology 1991,128:3055; Inagaki N y col.; Proceedings of the National Academy of Sciences USA 1994, 91:2679): the PACP type I receptor (PACAP-R-I) with equal affinity for PACAP-38 and PACAP-27, but which has an affinity 300 to 1000 times less for VIP; the PACAP type II receptor (PACAP-R-II) which recognises with the same affinity VIP, PACAP-38 and PACAP-27 and is thus denominated the common receptor of VIP-PACAP and corresponds to the receptor VIP VIP1-R, and the PACAP type III receptor (PACAP-R-III) which corresponds to the receptor VIP VIP2-R. Up until present there have been few studies on the biological actions of PACAP on the immune system. The effects of PACAP are often similar to those of VIP modulating the phagocyte function and proliferative responses.

DESCRIPTION OF THE INVENTION

[0006] The object of this invention is to develop preparation of VIP, PACAP and analogues thereof as therapeutic agents in the treatment of endotoxic shock.

[0007] The treatment consists of the administration to mammals in need thereof, of an effective quantity of an agent that inhibits the production of tumor necrosis factor (TNF) in an acceptable pharmaceutical vehicle.

[0008] VIP and PACAP have anti-inflammatory effects and inhibit the production of IL-1, IL-6 and TNF-, in animal models of the induction of endotoxic shock. As these cytokines play an important role in the development of said syndrome, VIP and PACAP can be used to regulate their production.

[0009] It is known that most of the effects of the endotoxic shock are mediated by activation of the immune system and the inflammatory mechanisms of the host as response to bacterial products. Macrophages play a key role in this process as after their activation factors such as nitric oxide, prostaglandins and cytokines responsible for symptoms such as fever, hypotension, disseminated micro-coagulation, multiple organ failure and finally death, are produced. Similarly, high levels of circulating TNF, IL-1 and IL-6 associated with endotoxemia have been described. In animal models these symptoms are reproduced both by administration of bacterial endotoxins (LPS) and by injection of TNF and IL-1. Other studies have underlined the diagnostic value in terms of the probability of survival represented by the levels of circulating IL-6.

[0010] The tumor necrosis factor (TNF) is produced by several types of cell that include monocytes and macrophages, T and B lymphocytes, neutrophils, mast cells, tumorous cells and fibroblasts. It is an important regulatory factor in other pro-inflammatory cytokines, such as IL-1 β , IL-6 and IL-8. TNF α induces the expression of adhesion molecules in endothelial cells, activates leukocytes to destroy the microorganisms, acts on the hepatocytes to increase the synthesis of serum proteins which contribute to the acute phase response and activate the coagulation system. Overproduction

thereof leads to immunopathologic diseases, autoimmunity and inflammation.

[0011] IL-6 is a multi-functional cytokine produced both by lymphocytes and by non-lymphoid cells. It regulates several aspects of the immune response, such as the production of proteins that mediate acute phase and hematopoiesis. Furthermore, it acts as a mediator in inflammatory response. Its production is regulated by several factors, which include TNF α , IL-1 and bacterial endotoxin (LPS).

[0012] Strategies of neutralisation of these cytokines have been tested in the treatment of endotoxic shock but the results do not indicate that there is a greater long-term survival. A treatment that inhibits the production of TNF and IL-6 would represent a considerable improvement in the evolution of endotoxic shock and in the probabilities of survival. Administration of VIP and PACAP in animal models achieves these effects and our invention consists of using a treatment with these neuropeptides for increasing the survival in cases of endotoxic shock.

DESCRIPTION OF THE FIGURES

[0013]

Figure 1 represents the production of TNF α by murin macrophages in culture (5×10^5 cells/ml) stimulated with 10ngr/ml of LPS in presence or absence of 10^{-8} M of VIP or PACAP over a period of 24 hours.

Figure 2 represents the production of TNF α by murin macrophages in culture (5×10^5 cells/ml) after 6 hours of culture with 10ngr/ml of LPS and to which 10^{-8} M of VIP or PACAP is added at different times.

Figure 3 represents the production of IL-6 by murin macrophages in culture (5×10^5 cells/ml) stimulated with 10ngr/ml of LPS in presence or absence of 10^{-8} M of VIP or PACAP over a period of 24 hours.

Figure 4 represents the production of IL-6 by murin macrophages in culture (5×10^5 cells/ml) after 6 hours of culture with 10ngr/ml of LPS and to which 10^{-8} M of VIP or PACAP is added at different times.

Figure 5 shows the Northern blot analysis for the presence of mRNA corresponding to TNF α and IL-6 in macrophages stimulated with LPS in presence or absence of VIP or PACAP (18S represents the corresponding rRNA as a control of the total amount of RNA load).

Figure 6 represents the survival of mice injected with 400 μ g. of LPS and, either simultaneously or after 30 minutes, 1 or 4 hours, with 5nmol. of VIP or PACAP. A. Control; B: VIP a 0 h.; C: VIP a 0.5 h; D:

VIP a 1 h.; E: VIP a 4 h.;

EMBODIMENT OF THE INVENTION

[0014] The examples that follow are only to illustrate the results obtained and do not limit the use of the invention. This use is laid out in detail in the specified claims.

EXAMPLE 1

VIP and PACAP inhibit the production of TNF α in macrophages stimulated with LPS

[0015] In experiments carried out *in vitro* VIP and PACAP inhibit the production of TNF α in peritoneal murin macrophages stimulated with LPS. The highest degree of inhibition reaches levels near to 60 % and occurs with doses of stimulation between 1 and 10 ng/ml of LPS. The IC₅₀ is around 80 pM, both for VIP and for PACAP and the effect was observed until the end of the experiment (see Figure 1). The inhibitory effect is the same if both neuropeptides are added up until 1 hour after stimulating the macrophages with LPS, although it reduces progressively until disappearing if added after 4 hours (see figure 2).

EXAMPLE 2

VIP and PACAP reduce the levels of circulating TNF α after injection with LPS

[0016] In an experiment carried out with mice the levels of circulating TNF α 2 hours after injection of 25 μ gr. of LPS approach 4 ngr./ml. Simultaneous administration of 5nmol of VIP or PACAP reduced said levels by 60%.

EXAMPLE 3

VIP and PACAP inhibit the production of IL-6 in macrophages stimulated with LPS

[0017] In experiments carried out *in vitro* VIP and PACAP inhibit the production of IL-6 in peritoneal murin macrophages stimulated with LPS. The highest degree of inhibition reaches levels near to 90% and occurs with doses of stimulation of 10 ngr./ml of LPS. The IC₅₀ is 8.6 pM, both for VIP and for PACAP and the effect was observed until the end of the experiment (see Figure 3). The effect is also observed if the neuropeptides are added after stimulation with LPS, although the degree of inhibition is progressively less (see Figure 4).

EXAMPLE 4

VIP and PACAP reduce the levels of circulating IL-6 after injection with LPS

[0018] In an experiment carried out with mice the levels of circulating IL-6 2 hours after injection of 25 μ gr. of LPS approach 1.5 ngr./ml. Simultaneous administration of 5nmol of VIP or PACAP reduced said levels by 60% and 75% respectively.

EXAMPLE 5

VIP and PACAP regulate the production of TNF α and IL-6 at a transcriptional level

[0019] Mouse macrophages were submitted to the experimental conditions of examples 1 and 3 and their mRNA isolated. This was then analysed using the Northern Blot technique to detect mRNA of TNF α and IL-6. Figure 5 shows the absence of transcripts for TNF α or IL-6 when the macrophages activated with LPS are also exposed to VIP or PACAP.

EXAMPLE 6

VIP and PACAP protect against the lethal effects of LPS

[0020] An experiment was carried out in which the survival over a 4-day period was studied of mice that had been injected with 400 μ gr. of LPS. The results are shown in Figure 6. The mortality in these circumstances was 100 % after 36 hours. With simultaneous administration of 5 nmol. of VIP or PACAP a survival rate of 60 % was attained at the end of the experiment. Administration of neuropeptides up to 1 hour after injection with LPS still gave survival rates near to 50%.

Claims

1. A method for the treatment of endotoxic shock in mammals characterised in that it comprises administration of an effective amount of an agent that inhibits the production of tumor necrosis factor (TNF) in a pharmaceutically acceptable vehicle.
2. A method for the treatment of endotoxic shock in mammals according to claim 1, characterised in that the inhibitory agent is the vasoactive intestinal peptide (VIP) or a fragment thereof or an analogue derived therefrom.
3. A method for the treatment of endotoxic shock in mammals according to claim 1, characterised in that the inhibitory agent is the peptide activating hypofissary adenylyate cyclase (PACAP) or a fragment thereof or an analogue derived therefrom.

4. A method for the treatment of endotoxic shock in mammals characterised in that it comprises the administration of an effective amount of an agent that inhibits the production of interleukin 6 (IL-6) in a pharmaceutically acceptable vehicle. 5
5. A method for the treatment of endotoxic shock in mammals according to claim 4, characterised in that the inhibitory agent is the vasoactive intestinal peptide (VIP) or a fragment thereof or an analogue derived therefrom. 10
6. A method for the treatment of endotoxic shock in mammals, according to claim 4, characterised in that the inhibitory agent is the peptide activating hypofissary adenylate cyclase (PACAP) or a fragment thereof or an analogue derived therefrom. 15

20

25

30

35

40

45

50

55

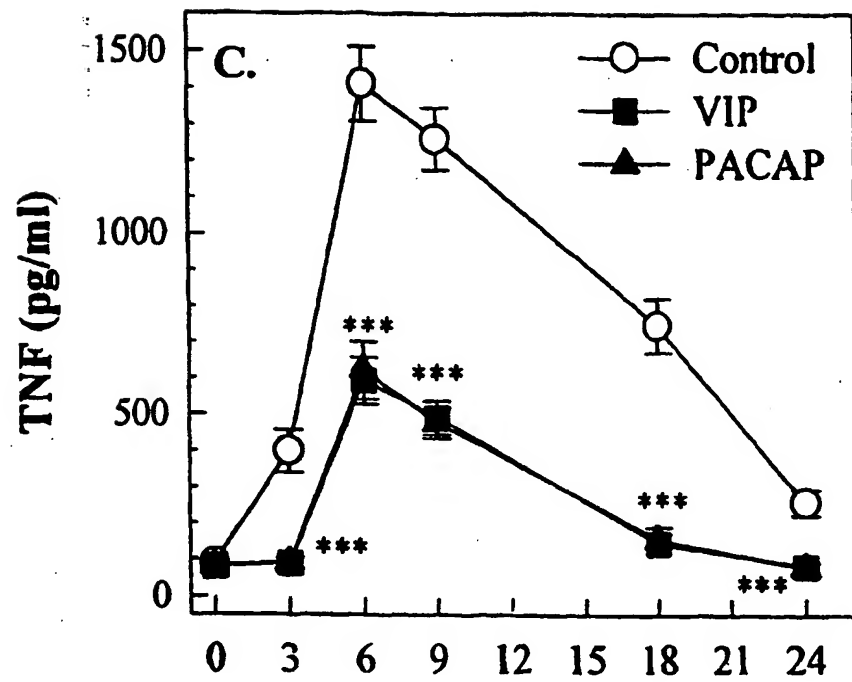
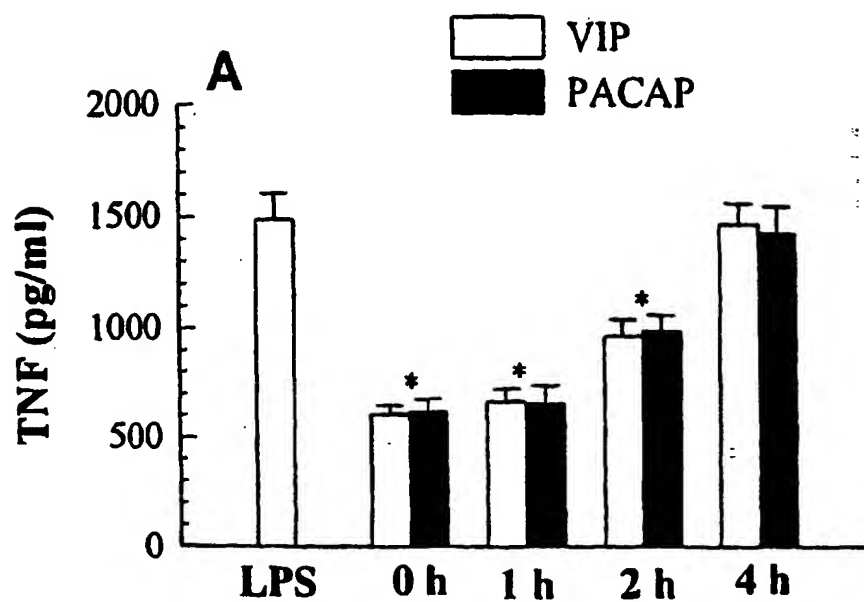


FIGURA 1

**FIGURA 2**

BEST AVAILABLE COPY

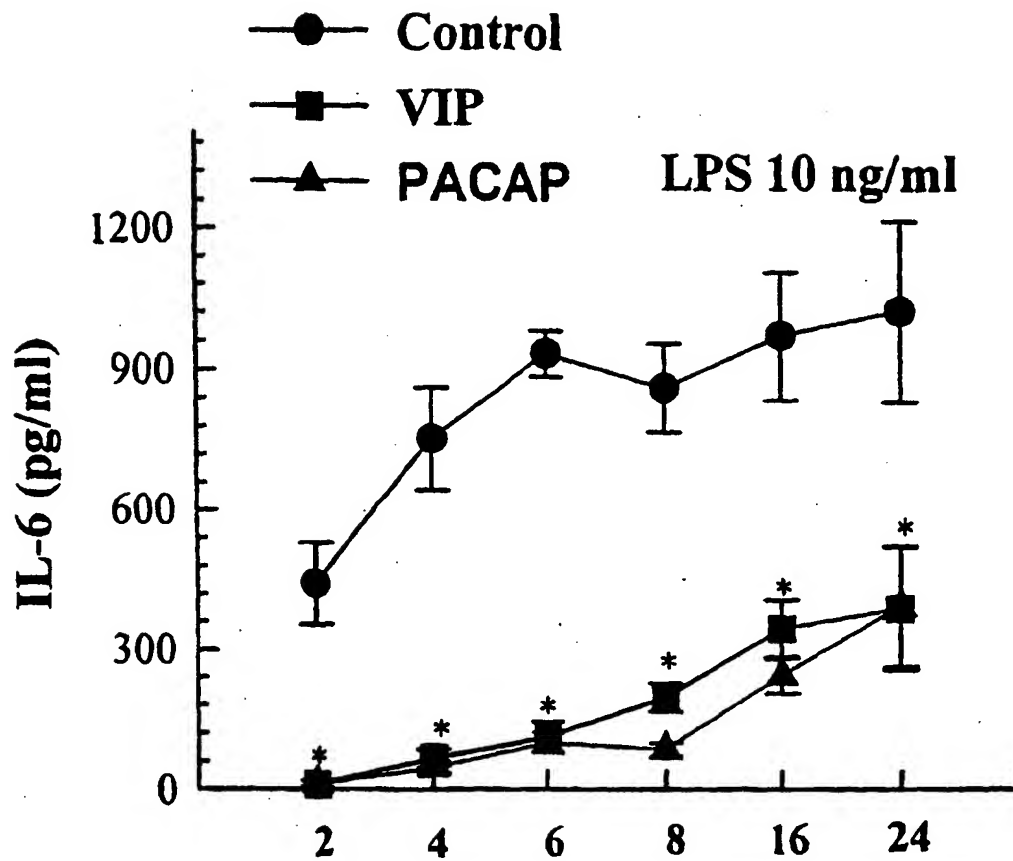


FIGURA 3

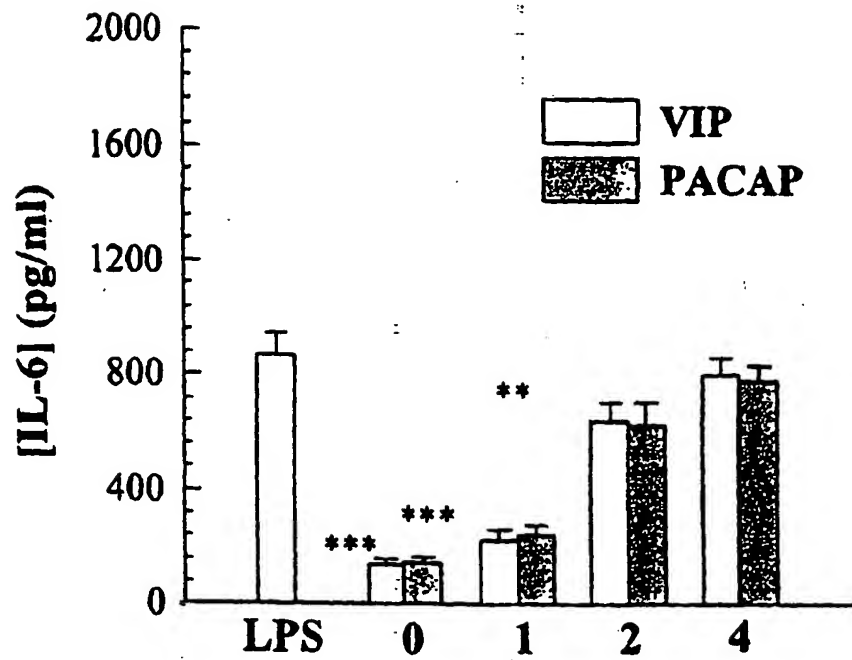


FIGURA 4

BEST AVAILABLE COPY

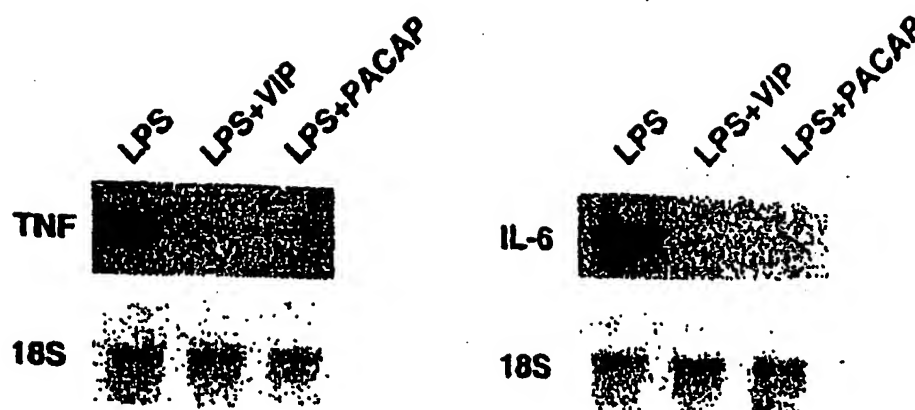


FIGURA 5

BEST AVAILABLE COPY

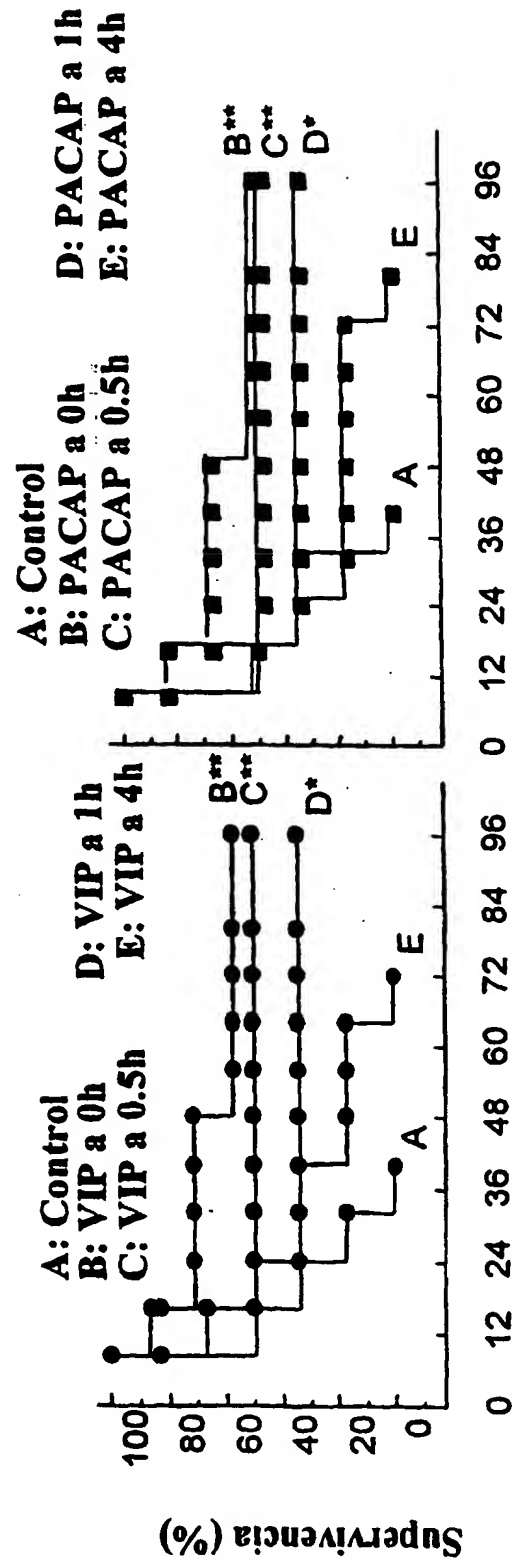


FIGURA 6

INTERNATIONAL SEARCH REPORT

International application No
PCT / ES 99 / 00101A. CLASSIFICATION OF SUBJECT MATTER ⁶:
CIP 6 A61k 38/22

According to International Patent Classification (IPC) or to both national classification and IPC6

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
CIP 6 A61K, C07KDocumentation searched other than minimum documentation to the extent that such documents are included in the fields searched
EPODOC, CA, BIOSIS, MEDLINE, MEDLINE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ⁸	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9620926 A1 (CELGENE CORPORATION); 11 July 1996 (11.07.96), abstract.	1
X	VERHOEF, J. et al. "The role of cytokines in Gram-positive bacterial shock". TRENDS IN MICROBIOLOGY. Vol.3, n°4, April 1995, pages 136-140, The whole document.	1,4
X	BRONER, C. W. et al. "Cyclic nucleotides and vasoactive intestinal peptide production in a rabbit model of Escherichia coli septicemia". THE AMERICAN JOURNAL OF THE MEDICAL SCIENCES. Vol: 309, n° 5, May 1995, pages 267-277, The whole document	2,5
X	YOSHINOBU, S. et al. "Anti-shock effect of pituitary adenylate cyclase activating polypeptide (PACAP) on experimental endotoxial shock in dogs". LIFE SCIENCES, Vol. 54, n° 22, 1994, pages 389-394, The whole document.	3,6



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A" document member of the same patent family

Date of the actual completion of the international search
19 July 1999 (19.07.99)Date of mailing of the international search report
29 July 1999 (29.07.99)

Name and mailing address of the ISA/

S. P. T. O

Facsimile No.

Authorized officer

Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

BEST AVAILABLE COPY

INTERNATIONAL SEARCH REPORT

International application No.
PCT/ ES 99/00101

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-5
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

BEST AVAILABLE COPY